Differential responses of soil ammonia-oxidizing archaea and bacteria to temperature and depth under two different land uses

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ABSTRACT

Although soil nitrification has been studied intensively, with an effort to elucidate the relative contributions of both ammonia-oxidizing archaea (AOA) and bacteria (AOB), their relative contributions to N_2O production remain unclear. Understanding the temperature- and depth-dependent activities of AOA and AOB, as well as production of N_2O, is of great importance for predicting their responses to climate change. This work applied the recently discovered AOB inhibitor, octyne, to soil microcosms incubated at different temperatures (20, 30, 40 °C) in order to differentiate ammonia-oxidation potential and N_2O production by AOA and AOB, in soils from different land uses and depth. Our results showed that surface soils (0-15 cm) possessed significantly greater ammonia oxidation potential than subsurface soils (30-45 cm) at all temperatures tested, and that AOA-associated nitrification potential dominated at higher temperatures for both summer and autumn soils. The accumulation of N_2O was only detected in surface agricultural soil at 30 °C and positively correlated with nitrite accumulation within the incubation period. The detected N_2O production, along with most nitrification potential activity, were attributed to AOB, implicating AOB as major producers of this greenhouse gas in the tested agricultural soil. Higher ammonia-oxidation activity and N_2O production within surface agricultural soil reinforces the importance of agricultural surface soils as sources of nitrification and N_2O production, with potential implications for land management practices and responses to climate change.

1. Introduction

Nitrification is traditionally considered a two-step microbially driven process for oxidizing ammonia (NH_3) to nitrate (NO_3^-) via nitrite (NO_2^-). This process links reduced and oxidized nitrogen pools by the combined activities of ammonia-oxidizing bacteria (AOB; Prosser, 1990), ammonia-oxidizing archaea (AOA; Könneke et al., 2005), nitrite-oxidizing bacteria (NOB; Prosser, 1990), and the newly discovered complete ammonia oxidation "comammox" bacteria (Daims et al., 2015; van Kessel et al., 2015).

Although ammonia oxidation to nitrite has been studied intensively, the relative contributions of AOA and AOB to this process and factors that may influence their contributions are still unclear (Hatzenpichler, 2012; Prosser and Nicol, 2012; Schleper, 2010). The isolation of AOA and AOB cultures from different environments suggests that temperature may have a role in niche separation between AOA and AOB. Specifically, cultured AOA range from mesophiles to hyperthermophiles, with optimum growth temperatures ranging from 25 °C for Candidatus Nitrosotalea devanaterra Nd1 (Lehtovirta-Morley et al.,

2014) to 72 °C for Candidatus Nitrosocaldus yellowstonii (de la Torre et al., 2008), with others in between these extremes (Daebeler et al., 2018; Jung et al., 2011, 2014a; Kim et al., 2012; Lehtovirta-Morley et al., 2016; Sauder et al., 2017; Stieglmeier et al., 2014a; Abby et al., 2018). Isolated AOB appear to have a narrower range, with the optimum temperatures for most strains ranging from 20 to 30 °C (Avrahami and Bohannan, 2007; Avrahami and Conrad, 2005; Groeneweg et al., 1994; Jiang and Bakken, 1999), but with growth of several also possible at 4 °C (Jones et al., 1988).

The influence of temperature on AOA and AOB community composition (Tourna et al., 2008) and activity (Horak et al., 2013; Taylor et al., 2017; Wu et al., 2013) has been assessed for various environmental samples. In soil, an increase of AOA-associated activity was observed for an agricultural soil in the warmer seasons of late summer and early fall (Taylor et al., 2012), with differential inhibition during soil incubations indicating that AOA possessed at least a 10 °C higher optimal temperature than AOB (Ouyang et al., 2017; Taylor et al., 2017). Whether these patterns are observed in contrasting land-use types and at different soil depths remains unclear. In addition, the effect

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of temperature on AOA- and AOB-associated nitrous oxide (N_2O) production associated with soil nitrification is poorly understood. Because previous studies examining the effects of temperature on N_2O production (Avrahami et al., 2003; Conrad et al., 1983; MacDonald et al., 1997; Mogge et al., 1998, 1999; Slemr et al., 1984) were conducted before the discovery of AOA, and a recent study only focused on greenhouse agricultural soils (Duan et al., 2018), the temperature-dependent relative contributions of AOA and AOB to soil N_2O production activity in a natural system requires further investigation.

Nitrous oxide, a trace gas with a ~300 fold greater global warming potential than CO_2, is a reactant capable of causing stratospheric ozone destruction (Ravishankara et al., 2009). Soil is considered the largest source of N_2O emissions (Schreiber et al., 2012; Syakila and Kroeze, 2011), of which microbial ammonia oxidation contributes approximately 80% (Gödde and Conrad, 1999; Kool et al., 2011; Wrage et al., 2001; Zhu et al., 2013). AOB produce N_2O enzymatically through two mechanisms: incomplete oxidation of hydroxylamine (NH_2OH) to NO_2^- and sequential reduction of NO_2^- to NO and N_2O by "nitrifier-denitrification" (Arp and Stein, 2003). Although the N_2O-producing mechanism within AOA has not been fully resolved, it is thought that AOA produce N_2O during NH_3 oxidation through an abiotic reaction between NH_2OH and NO, an intermediate of the AOA ammonia oxidation pathway (Stieglmeier et al., 2014b), which has been demonstrated for pure cultures (Jung et al., 2011; Kozlowski et al., 2016; Qin et al., 2017; Stieglmeier et al., 2014b). AOA-associated N_2O production in environmental samples has been reported in several studies (Giguere et al., 2017; Hink et al., 2017, 2018; Löscher et al., 2012; Peng et al., 2016; Santoro et al., 2011). Although some studies have determined the relative contributions of AOA and AOB to soil nitrification using selective inhibitors (Daebeler et al., 2015; Duan et al., 2018; Giguere et al., 2015; Lu et al., 2015; Ouyang et al., 2016; Taylor et al., 2013, 2010), and examined the relative contributions of AOA and AOB to nitrifier-dependent N_2O production in agricultural or non-cropped soils (Shi et al., 2017; Giguere et al., 2017; Hink et al., 2017; Wang et al., 2016), no microcosm study has yet examined the influence of temperature on N_2O production by AOA and AOB along a soil depth profile.

In our previous work, we characterized the influence of depth and land-use on thaumarchaeotal and bacterial community structures at the **rare** Charitable Research Reserve (Lu et al., 2017; Seuradge et al., 2017), showing depth- and land-use-associated community changes. Using this knowledge of thaumarchaeotal and bacterial biogeography, site-specific heterogeneity, and depth profiles, the aim of this study was to perform a targeted investigation into how AOA and AOB at different soil depths and land uses respond to temperature with respect to their relative contributions to ammonia oxidation and N_2O production.

2. Material and methods

2.1. Site selection and soil sampling

Soil samples were taken from the **rare** Charitable Research Reserve (Cambridge, Ontario) in early September ("summer" samples) and early November ("autumn" samples) in 2015. Soil samples were collected at two depths (0-15 and 30-45 cm) from either an agricultural site (Preston Flats; A) or forest site (Hogsback; F). According to our previous research, sites A and F possessed distinct bacterial and thaumarchaeotal communities that also varied with depth (Lu et al., 2017; Seuradge et al., 2017). A composite soil sample from each depth at each site was generated by randomly collecting 3-5 replicates from the same location as our previous sampling plots used for a bacterial and thaumarchaeotal biogeography survey (Lu et al., 2017; Seuradge et al., 2017). The agricultural site has been under no-till management since 2002, operated under a rotation of corn (*Zea mays*) and soybean (*Glycine max*) from 2002 to 2011, and a corn monocrop since 2011. The forest site is a mixture of northern hardwood and Carolinian tree species, maintained as pristine forest for over 100 years, and is thus classified as a mature

forest. Soils from both sites are classified as Burford series, which is typical for the area along the Grand River. The soils are well-drained and calcareous, with a relatively thin A horizon. The B horizons of both soils have a sandy clay loam texture, wavily extending into a C horizon that contained over 50% gravel (Presant and Wicklund, 1971). Soil samples for the incubation experiment were sieved (4.75 mm) and stored at 4 °C prior to establishing microcosms, or at -20 °C prior to physicochemical analysis at the Agriculture and Food Laboratory (University of Guelph).

2.2. Whole soil nitrification assay

Similar to a previously published method (Lu et al., 2015), a modified 8-day whole soil assay was used to measure the nitrification potential (NP) attributed to AOA and AOB in composite soil samples. Triplicate subsamples from each composite soil were pre-incubated for two days in a 120-mL serum bottle (15 g field moisture soil per replicate) at room temperature (22 °C) with a loosely capped stopper prior to microcosm incubation. This room temperature pre-incubation minimized the influence of 4 °C storage (Giguere et al., 2015). An NH_4Cl solution was added to each microcosm, resulting in a final concentration of 200 mg-N kg^{-1} $soil_{dry}$ and a gravimetric water content of 30%. Serum bottles were sealed with silica stoppers and incubated in the dark at 20 °C, 30 °C, or 40 °C. At the beginning of the incubation, triplicate bottles were amended with either acetylene (0.02% v/v) or octyne gas (1.9% v/v) following a protocol published elsewhere (Taylor et al., 2013). Acetylene was used to irreversibly inactivate ammonia monooxygenase of both AOA (Offre et al., 2009; Vajrala et al., 2013) and AOB (Hyman and Wood, 1985), whereas octyne is a specific inhibitor of AOB only (Giguere et al., 2015, 2017; Hink et al., 2017; Lu et al., 2015; Taylor et al., 2013, 2015). To monitor net nitrification potential, the incubated soils were sampled at 0, 2, 4, 6, and 8 d by removing 2.5 g of soil, and then re-established inhibitor concentrations. Nitrification potential rates were determined by measuring the accumulation of $NO_2^- + NO_3^-$ at each sampling point (Taylor et al., 2010). Total net nitrification potential rates were determined by the accumulation of $NO_2^- + NO_3^-$ without inhibitors (i.e., acetylene or octyne). Nitrification potential in octyne-amended microcosms was attributed to AOA, with the difference between no inhibitor and octyne-amended microcosms attributed to AOB activity. To determine $NO_2^- + NO_3^-$ production from autotrophic nitrification in the forest soil, $NO_2^- + NO_3^-$ concentrations in acetylene-amended microcosms at each time point were subtracted from those in uninhibited microcosms, as described previously (Lu et al., 2015). Because forest soil microcosms showed a decrease in $NO_2^- + NO_3^-$ concentrations after six days of incubation, indicating a possible increase in denitrification activity and/or immobilization (data not shown), only the first six days of data were used for analysis.

2.3. Analysis of N_2O, NO_2^- and $NO_2^- + NO_3^-$

N_2O concentrations were determined in the headspace of microcosms immediately before each sampling point, as described previously (Coyotzi et al., 2017), with 1 mL gas sampled using a disposable syringe and injected into a GC-2014 gas chromatograph (Shimadzu Scientific Instruments, Columbia, MD) equipped with a Porapak Q 80-100 column and an electron capture detector (ECD).

Soil NO_2^- concentrations were measured directly with Griess reagents, whereas soil $NO_2^- + NO_3^-$ accumulation was determined using a vanadium reduction assay to convert NO_3^- to NO_2^- before adding Griess reagents to measure NO_2^- (Miranda et al., 2001; Sauder et al., 2017). All assays were conducted in clear flat bottom 96-well plates (Greiner, Frickenhausen, Germany) and absorbance measured at 550 nm using a Filtermax F5 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA).

2.4. DNA extraction and qPCR

Soil genomic DNA was extracted using the PowerSoil DNA Isolation Kit (MO BIO, Carlsbad, CA) according to the manufacturer's instructions. Ammonia-oxidizing archaea (AOA) and bacteria (AOB) were quantified by determining *amoA* gene abundance using recently developed primer sets GenAOAF/R and GenAOBF/R, respectively (Meinhardt et al., 2015). All qPCR amplifications used SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA) and were performed on a CFX96 Real-Time PCR Detection system (Bio-Rad, Hercules, CA). All qPCR amplifications were carried out in duplicate 10 μL volumes. Each reaction contained 5 μL of 2 × SsoAdvanced Universal SYBR Green Supermix, 1 μL of template DNA, 0.4 μL of a 10 mg mL^{-1} bovine serum albumin solution, and 0.025 μL of each primer at 100 μM concentrations. Thermal cycling conditions were as follows: 3 min of initial denaturation at 98 °C, followed by 39 cycles of denaturation at 98 °C for 15 s and annealing/extension at 55 °C for 30 s. The standard curves for both AOA and AOB *amoA* genes were constructing with 10-fold serial dilutions of PCR amplicons generated from the same soil samples with the same primers. Efficiencies were 91.2% and 95.8% for AOA and AOB *amoA* qPCR data, respectively. Standard curve R^2 values were all > 0.99.

2.5. Statistics

A three-way ANOVA (analysis of variance) was used to identify the effects of land-use, depth, and temperature on activity measurements (NO_2^- + NO_3^- or N_2O accumulation), followed by a Tukey's test. Two-way ANOVA was used to access the influence of depth and temperature on NO_2^- accumulation. Spearman's rank order correlation was used to test the relationship between environmental factors and activity measurements or ammonia oxidizer gene abundances. All statistical tests were carried out using R 3.2.3 (R Core Team, Vienna).

3. Results

3.1. Soil sample characteristics

From collected soil metadata (Table 1), we observed that measured NH_4^+ concentrations were influenced by land-use, with higher concentrations in composites from forest soil than those from agricultural soil. The NO_3^- concentrations were influenced by depth, higher for subsurface agricultural composites than subsurface forest soil composites. The pH of the soil samples spanned a relatively narrow range (pH 6.7–8.2). The C:N ratio was highest among subsurface soil samples collected from forest sites in autumn.

3.2. Dynamics of NO_2^- + NO_3^- accumulation

Background soil nitrification rates were determined for all soil samples, without additional NH_4^+ at 30 °C, by measuring net accumulation of NO_2^- + NO_3^-. The background nitrification rate was highest (1.44 mg N kg^{-1} soil d^{-1}) in autumn surface agricultural soils across all samples (Figure S1). All subsurface soils showed nitrification rates below 0.20 mg N kg^{-1} soil d^{-1}. In all soils, background nitrification rates were attributed primarily to AOA, with octyne-resistant activity ranging from 63 to 100% of the uninhibited samples (Figure S1). Acetylene-resistant activity was not observed for any soil samples.

When additional NH_4^+ was added to soil samples, detectable NO_2^- + NO_3^- did not accumulate during the six-day incubation time for agricultural soil microcosms in the presence of acetylene, indicating that the accumulation of NO_2^- + NO_3^- in acetylene-free microcosms was dominated by autotrophic nitrifiers. However, a relatively small amount of acetylene-resistant activity (up to 9.2% in surface soil and 12.2% in subsurface soil) was detected in autumn forest soils after four days of incubation, suggesting heterotrophic nitrification. The overall nitrification potential (NP) ranged from 0.72 ± 0.16 to 6.73 ± 0.11 mg N kg^{-1} soil d^{-1} for summer surface soils and 0.18 ± 0.03 to 1.88 ± 0.12 mg N kg^{-1} soil d^{-1} for summer subsurface soils (Fig. 1A and B), and 0.22 ± 0.05 to 8.46 ± 0.48 mg N kg^{-1} soil d^{-1} for autumn surface soils and 0.04 ± 0.02 to 0.24 ± 0.11 mg N kg^{-1} soil d^{-1} for autumn subsurface soils (Fig. 1E and F). No NP was detected in the subsurface forest soil (autumn) replicates incubated at 40 °C (Fig. 1F and H). The overall NP was influenced by land use type ($p < 0.001$), depth ($p < 0.001$), and temperature ($p < 0.001$). Interactions among the three factors were detected by a three-way ANOVA and, as a result, the overall NP was analyzed independently for each soil type. In general, agricultural soils had a higher overall NP than forest soil, and surface soils higher than subsurface soils, at the same incubation temperature (Fig. 1A, B, E, and F). Surface soils under both land uses reached greatest activity at 30 °C for soils collected in both seasons but statistical analysis indicated no significant difference between nitrification activity measured at 30 and 40 °C ($p = 0.18$), nor between 20 and 40 °C ($p = 0.13$) among agricultural soils collected in autumn. For subsurface soils, higher nitrification activity was observed at 20 °C across autumn agricultural and forest site samples, but not significantly different from other temperatures ($p > 0.05$). Agricultural subsurface soils collected in summer reached highest activity at 30 °C ($p < 0.01$), and its NP was even higher than summer forest surface soils incubated at 40 °C ($p < 0.01$).

The AOA-associated (octyne-resistant) NP increased with incubation temperature in surface soils for both agricultural and forest samples collected in autumn (Fig. 1G and H). In autumn agricultural soil, the lowest and highest rates were observed at 20 and 40 °C for surface samples, respectively (Fig. 1G). Within surface forest soils, the highest AOA-associated NP was 0.55 ± 0.22 mg N kg^{-1} soil d^{-1} (Fig. 1H), which was only 8.8% of highest rate observed for the agricultural soil. No statistical difference was detected among AOA-associated NP in subsurface soils (autumn) from either agricultural or forest sites, respectively. Agricultural soils collected in summer showed different temperature responses in terms of AOA-associated activity. The lowest AOA-associated activity for surface soil was detected at 30 °C, with similar NP as that of autumn samples at 20 °C (Fig. 1C). Similar to autumn samples, no significant difference was detected among forest

Table 1
Properties of soil samples from active agricultural (A) and forest (F) sites. Soils were composites of field replicates.

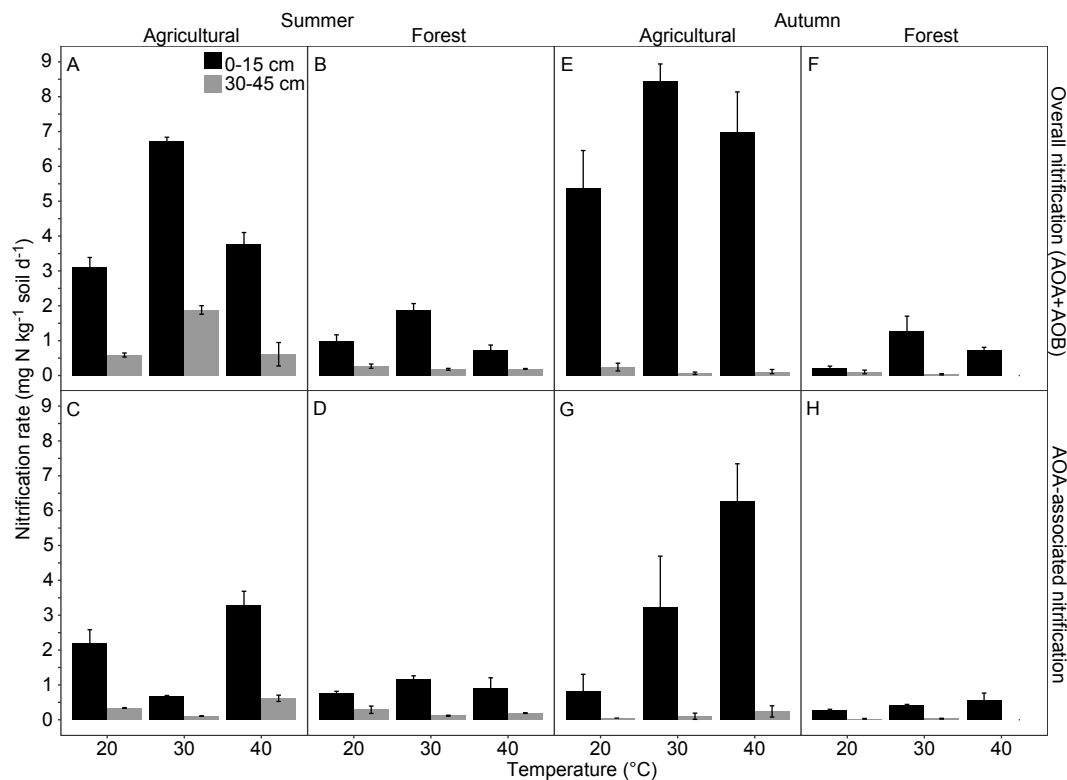| Site	Sampling season	Depth (cm)	NH_4^+ (mg N kg soil^{-1})	NO_3^- (mg N kg soil^{-1})	Inorganic C (%)	Organic C (%)	Total N (%)	C:N ratio	pH
A	Summer	0–15	1.22	6.85	1.25	1.59	0.12	23.7	8.0
		30–45	0.60	3.95	0.29	1.21	0.08	18.8	7.7
F		0–15	10.6	14.3	0.00	5.94	0.33	18.0	6.8
		30–45	8.70	2.98	2.66	1.55	0.08	52.6	7.7
A	Autumn	0–15	0.59	9.41	0.85	1.90	0.13	21.2	7.8
		30–45	0.63	1.53	0.40	0.60	0.05	20.0	7.9
F		0–15	3.39	9.14	0.00	2.89	0.16	18.1	6.7
		30–45	0.95	0.62	5.91	0.47	0.05	127.6	8.2

Fig. 1. Overall nitrification potential (A, B, E, and F) and AOA-associated nitrification potential (C, D, G and H) of a six-day whole soil incubation for both agricultural (A and C, E and G) and forest (B and D, F and H) soils at three different incubation temperatures (20, 30, and 40 °C) and depths (0-15 and 30-45 cm). Summer (A-D) and autumn (E-H) soils are included. The NP was measured as nitrite and nitrate accumulation per gram of soil per day. No activity was detected in subsurface forest soils collected in autumn.

surface soils collected in summer under different temperatures ($p > 0.05$; Fig. 1D).

In order to evaluate the relative contribution of AOA to total (AOA- and AOB-associated) nitrification, the proportion of AOA-supported nitrification to total nitrification activity was calculated (Fig. 2). AOA relative activity ranged from ∼ 5 to 98% across all microcosms from the agricultural and forest sites, and across multiple depths and temperatures (Fig. 2). When incubated at 40 °C, nitrification in all soils was dominated by AOA (mean percentage > 50%), except for autumn subsurface forest soils where no activity was detected. The AOA-associated activity was 100% in surface forest soil collected in summer (Fig. 2A) and subsurface agricultural soil collected in autumn (Fig. 2D). At 20 °C, agricultural soils and subsurface forest soil replicates were dominated by AOB-associated nitrification among autumn samples, but switched to AOA-dominance in summer samples. The only two summer soil samples with activity dominated by AOB were agricultural soils from both depths incubated at 30 °C (Fig. 2A and B). Furthermore, two-way ANOVA showed that the proportion of AOA-associated activity for agricultural soils was influenced significantly by temperature ($p = 0.003$ summer soils, $p < 0.001$ autumn soils) and depth ($p = 0.01$ summer soils, no significant difference among autumn soils). For AOA-associated forest soil proportions, no significant depth effects were detected for samples collected in both seasons, whereas temperature ($p < 0.001$) showed a significant influence on autumn samples, but not on summer samples. Three-way ANOVA detected significant land-use effect on AOA-associated activity percentage only among autumn samples ($p < 0.001$), whose effect became insignificant among summer samples ($p = 0.103$).

3.3. AOA and AOB amoA gene abundance

Overall AOA *amoA* gene abundance decreased with depth in all samples (Fig. 3), with significant differences only detected among

autumn samples ($p = 0.59$ summer soils, $p < 0.01$ autumn soils). AOB *amoA* gene abundance were similar at each depth among summer samples ($p = 0.98$), as well as autumn forest samples ($p = 0.83$). A significant decrease of AOB *amoA* gene abundance with increasing depth was only detected among autumn agricultural samples ($p < 0.01$). Temporal change was detected among AOA *amoA* gene abundance in subsurface soils from both land-use type ($p = 0.04$ agricultural soils, $p = 0.02$ forest soils), but no significant change was detected in surface soils. In contrast, AOB *amoA* gene abundance was relatively stable in both seasons, with no significant change detected in either surface or subsurface soils.

When correlated with environmental factors, summer and autumn samples showed a different trend. AOA *amoA* gene abundance decreased with pH ($p < 0.001$, rho = -0.85) and increased with soil NO_3^- content ($p < 0.001$, rho = 0.89) in autumn samples, but not in summer samples. Summer soil AOA *amoA* gene abundance increased with total carbon ($p = 0.04$, rho = 0.68). AOB *amoA* gene abundance decreased with total carbon ($p = 0.03$, rho = -0.69, autumn; $p = 0.02$, rho = -0.76, summer) and soil NH_4^+ content ($p = 0.03$, rho = -0.82, autumn), and increased with NO_3^- ($p = 0.02$, rho = 0.70, autumn) and pH ($p = 0.03$, rho = 0.71, summer).

3.4. N_2O production dynamics with $NO_2^- + NO_3^-$ accumulation

Across all microcosm incubations, N_2O production was only detected in surface agricultural soil replicates incubated at 30 °C. The overall six-day average N_2O production rate for autumn replicates was 0.02 mg N_2O-N kg soil^{-1} d^{-1}, and 0.14 mg N_2O-N kg soil^{-1} d^{-1} for summer. Proportional yields of N_2O were calculated as accumulation of N_2O-N divided by $NO_2^- + NO_3^-$-N accumulation. The six-day average yield for the whole soil incubation was 0.28 ± 0.14% for autumn samples and 2.18 ± 0.53% for summer samples (Table 2).

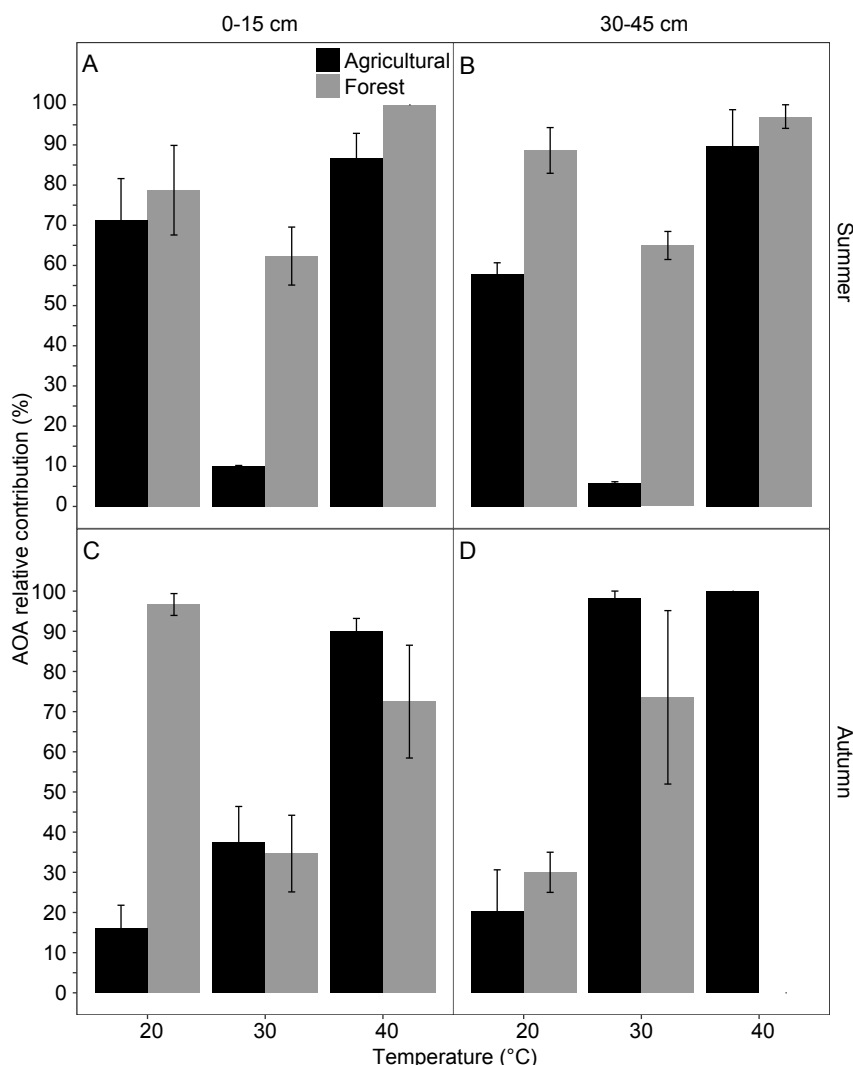By day six, 2.33 and 5.46 nmol g soil^{-1} N_2O was recorded in the

Fig. 2. Relative contribution of AOA to total nitrification activity in summer (A and B) and autumn (C and D) soil samples collected from both agricultural (black) and forest (grey) sites, incubated at three different incubation temperatures (20, 30, and 40 °C). Two depths were included: surface (0-15 cm; A and C) and subsurface (30-45 cm; B and D). The AOA contribution is calculated by dividing octyne-resistant activity over total activity. No activity was detected in subsurface forest soils collected in autumn.

acetylene-treated replicates for autumn and summer soils, respectively, which was likely not associated with nitrification because detectable levels of $NO_2^- + NO_3^-$ did not accumulate in the acetylene treatments in these agricultural soils. An additional 5.24 nmol N_2O g soil^{-1} production was measured in the absence of acetylene for autumn samples, which was inferred to be associated with nitrification. The N_2O concentration detected in summer soils in the absence of acetylene was 31.4 nmol N_2O g soil^{-1}, six times higher than that of autumn soils. Octyne-treated agricultural soil had no detectable N_2O production, indicating that AOA (octyne-resistant) were not responsible for the N_2O production. The six-day average N_2O yield for AOB only was 0.51 ± 0.36% for autumn soils and 2.43 ± 0.59% (Table 2) for summer soils. The six-day average N_2O production rate per cell was estimated to be 1.44×10^{-7} nmol cell^{-1} d^{-1} for AOB-supported nitrification in autumn soils and 8.84×10^{-7} nmol cell^{-1} d^{-1} for summer soils.

N_2O yields after 2, 4, and 6 days of incubation were calculated for both AOB-dominated (octyne-sensitive) and total activity (Table 2). The N_2O yield correlated significantly with incubation time ($p < 0.05$) for both AOB-dominated and total activity, indicating the production of N_2O together with $NO_2^- + NO_3^-$ accumulation was not independent of incubation time. The N_2O yields based on AOB-dominated were compared to total activity at each sampling point (days 2, 4, and 6), to

confirm that AOA did not contribute significantly to N_2O production. The statistical analysis did not detect any significant differences between N_2O yields for AOB-dominated and total activity at the three time points ($p > 0.05$).

3.5. Dynamics of NO_2^- accumulation and N_2O production

NO_2^- accumulation was detected only in surface and subsurface agricultural soil microcosms without any inhibitor (octyne or acetylene), collected either in summer and autumn (Fig. 4), with accumulation rates ranging from 0.002 to 5.82 mg N kg^{-1} soil d^{-1}. In surface agricultural soil microcosms, NO_2^- was detected after 2 days incubation, and the percentage of NO_2^- relative to total $NO_2^- + NO_3^-$ concentrations increased with incubation time reaching 45.9% and 68.8% in summer and autumn soil microcosms, respectively, after incubation at 30 °C for 6 days (Fig. 4A and C). In subsurface soil microcosms incubated at 30 °C, this proportion reached 100% after 4-and 6-days incubation in summer and autumn samples, respectively (Fig. 4B and D). NO_2^- accumulation was also observed in autumn surface agricultural soils incubated at 20 and 40 °C (Fig. 4C) and summer subsurface soils at 20 °C (Fig. 3B). Both temperature ($p < 0.001$) and incubation time ($p < 0.001$) influenced the NO_2^- proportion significantly in surface autumn samples (Fig. 4C).
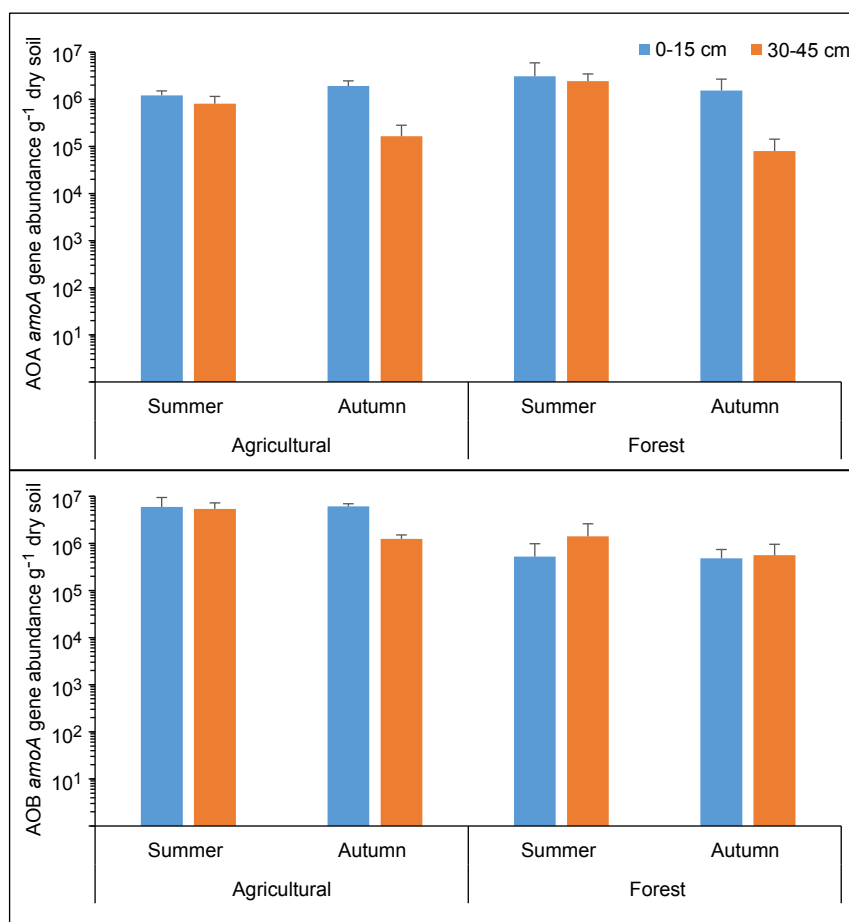
Fig. 3. Abundance of *amoA* genes from ammonia-oxidizing archaea (upper) and bacteria (bottom) in active agricultural and forest soils.

For surface agricultural soil microcosms where N_2O production was detected, NO_2^- accumulation started at 2.29 mg N kg^{-1} soil after 2 days incubation, and reached 18.5 mg kg^{-1} and 34.9 mg N kg^{-1} soil after 6 days incubation in summer and autumn soil microcosms, respectively. The N_2O accumulation in summer agricultural soils correlated significantly with NO_2^- accumulation ($R^2 = 0.67$, $p = 0.004$), but no significant correlation was observed in autumn agricultural soils ($R^2 = 0.13$, $p = 0.19$) (Fig. 5).

4. Discussion

4.1. Relative contributions of AOA and AOB to soil nitrification

We examined the effect of temperature on short-term (≤ 6 days) AOA-associated (octyne-resistant) and AOB-associated (octyne-sensitive) ammonia oxidation activity in surface (0-15 cm) and subsurface

(30-45 cm) agricultural and forest soils. At the same time, N_2O produced during ammonia oxidation was measured together with $NO_2^- + NO_3^-$ accumulation. Although several studies have reported the activity of AOA isolates at 35-72 °C (de la Torre et al., 2008; Lehtovirta-Morley et al., 2016; Tourna et al., 2011), the influence of temperature on *in situ* ammonia oxidation was not clear prior to this study.

During the soil microcosm incubations, additional NH_4^+ increased the octyne-sensitive (AOB-associated) contribution to overall activity compared to no addition, except for autumn subsurface agricultural soil and autumn subsurface forest soil. This NH_4^+-induced AOB activity was reported in several studies (Giguere et al., 2015; Hink et al., 2018; Jia and Conrad, 2009; Ouyang et al., 2016; Taylor et al., 2010, 2012; Xia et al., 2011). Absolute AOA-associated nitrification potential was higher within agricultural soil samples than forest soils at each incubation temperature (Fig. 1C, D, G, and H). Given that AOA

Table 2
Ammonia oxidation and N_2O production in surface agricultural soil incubated for 2, 4, or 6 days. All values are given mean with standard deviation (n = 3). Soils were composites of field replicates.

	Incubation time (day)	N_2O accumulation (mg N kg^{-1} soil)	Total $NO_2^- + NO_3^-$ accumulation (mg N kg^{-1} soil)	AOB $NO_2^- + NO_3^-$ accumulation (mg N kg^{-1} soil)	Total N_2O yield (%)[*]	AOB N_2O yield (%)[†]
Autumn soils	2	0.05 (0.05)	33.5 (6.07)	12.3 (3.96)	0.16 (0.21)	0.53 (0.72)
	4	0.13 (0.08)	38.5 (3.06)	26.3 (2.15)	0.36 (0.22)	0.51 (0.29)
	6	0.15 (0.08)	50.8 (2.87)	31.5 (6.25)	0.28 (0.14)	0.51 (0.36)
Summer soils	2	0.05 (0.02)	14.3 (0.72)	11.5 (1.08)	0.37 (0.12)	0.46 (0.13)
	4	0.53 (0.11)	22.3 (1.65)	19.6 (1.66)	2.43 (0.62)	2.77 (0.73)
	6	0.88 (0.21)	40.4 (0.64)	36.3 (0.57)	2.18 (0.53)	2.43 (0.59)

Percentage yields of N_2O were calculated as accumulation of N_2O-N divided by $NO_2^- + NO_3^-$-N accumulation. [*] Total N_2O yield was calculated by dividing N_2O accumulation by total $NO_2^- + NO_3^-$-N accumulation; [†] AOB N_2O yield was calculated by dividing N_2O accumulation over AOB $NO_2^- + NO_3^-$-N accumulation.

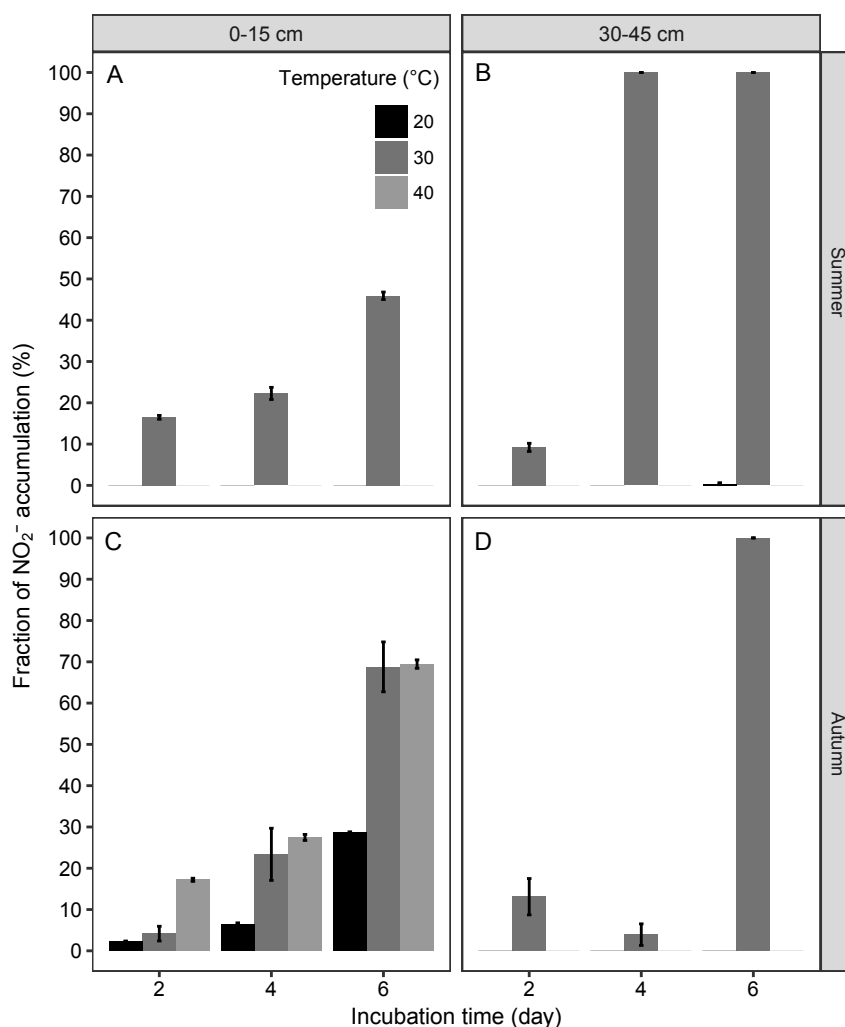**Fig. 4.** Fraction of NO_2^- in $NO_2^- + NO_3^-$ accumulation in agricultural surface (A and C) and subsurface (B and D) soils collected in summer (A and B) and autumn (C and D) at 20, 30, and 40 °C measured after 2, 4, and 6 days of incubation. NO_2^- accumulation in surface summer soil and subsurface autumn soil was detected only at 30 °C.

abundance was of the same order of magnitude among forest and agricultural soils at the same depth, this discrepancy between agricultural and forest soil AOA-associated ammonia oxidation rates is probably due to the differences in AOA community composition in these two soils.

4.2. Temperature effect on nitrification

In this study, temperature was identified as an important factor controlling the magnitude of AOA and AOB ammonia oxidation and N_2O production. We observed the highest AOA-associated and AOB-associated activity in surface soils at 40 °C and 30 °C, respectively. The highest AOA-associated activity in subsurface agricultural soils was recorded at 40 °C for both summer and autumn soils, whereas 20 °C and 30 °C favored AOB-associated activity in autumn and summer subsurface agricultural soil, respectively. In forest subsurface soils, the optimal temperature for AOB showed seasonal variation, 20 °C in autumn soil and 30 °C in summer soil, but 40 °C did not favor AOB activity in either soil. In general, our results showed that AOA were more active at higher temperature, whereas AOB preferred lower temperature. Several studies have reported a dominance of AOB for agricultural soil ammonia oxidation (Jia and Conrad, 2009; Ouyang et al., 2016; Xia et al., 2011), within which soil microcosms were carried out at temperatures ~27 °C, which agreed with our results at 30 °C for both surface agricultural and surface forest soils. Recently, Taylor et al. (2017) reported

that soil AOA and AOB contributed differently to ammonia oxidation across a temperature range of 4–42 °C, with AOA having optimal temperatures at least 12 °C higher than AOB. A similar pattern was observed in a slurry incubation with greenhouse agricultural soils covering 5-45 °C (Duan et al., 2018). Results from a slurry assay may not extrapolate to whole soil incubations because of disturbed homogenizing incubation and redox conditions, which can influence ammonia oxidation rates (Lu et al., 2015). However, a similar pattern was observed in this study, with AOA showing higher activity at elevated temperatures compared to AOB, as found previously by Taylor et al. (2017) and Gubry-Rangin et al. (2017). In addition, there may be a confounding influence of temperature with soil pH, which has been demonstrated to be a major factor determining thaumarchaeotal niche differentiation (Prosser and Nicol, 2012). A differential temperature response of the thaumarchaeotal community varying with soil pH was similar to that observed by Gubry-Rangin et al. (2017), indicating a pH-dependent selection mechanism on AOA populations with different optimal growth temperatures. The different optimal temperature of soil AOA along soil depth in this study also suggested the presence of potential different lineages along soil profile, both in forest and agricultural soils.

4.3. Temperature effect on soil N_2O production

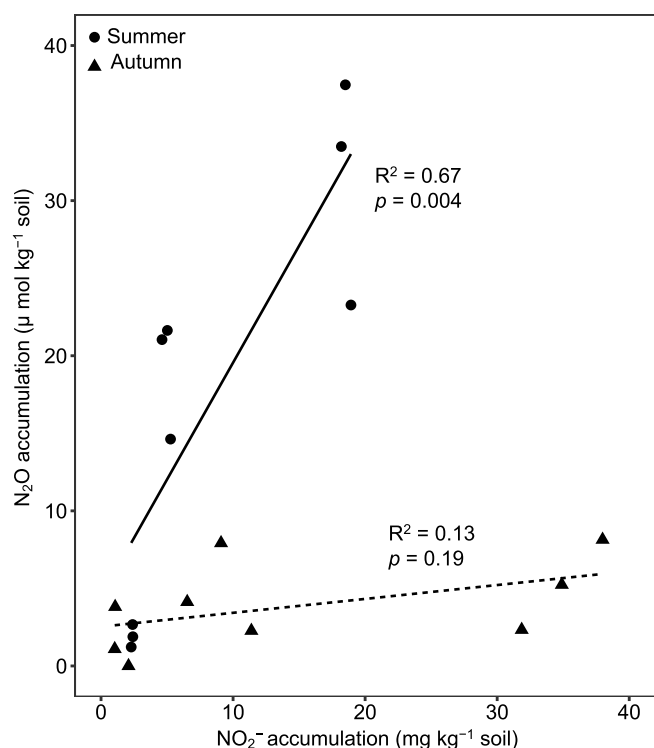The detection of N_2O production in surface agricultural soils only at

Fig. 5. Correlation between N_2O and NO_2^- accumulation in agricultural surface soils throughout 6 days incubation at 30 °C. Circles = summer soil, triangles = autumn soil. Significant ($p < 0.05$) correlation was only observed in summer soil.

30 °C provides evidence for a temperature-dependent influence on N_2O production via nitrification. However, it should be noted that an inability to detect the presence of N_2O at 20 or 40 °C does not necessarily mean the absence of N_2O under those conditions. The N_2O detection limit of the method used in this study was 0.05 nmol g^{-1} soil. Based on an agricultural soil AOA pure culture, whose N_2O yield is 0.08–0.23% (Jung et al., 2011, 2014b), the theoretical archaeal N_2O production at 40 °C would be ~0.18–0.52 nmol g^{-1} soil d^{-1} for autumn agricultural soils and 0.09–0.27 nmol g^{-1} soil d^{-1} for summer agricultural soils used in our study. This theoretical value is above the detection limit, so it is more likely that in the soil samples used here, the actual AOA-associated N_2O production was not as high as in pure cultures. Such a low *in situ* N_2O production rate by AOA was also reported by Hink et al. (2018, 2017) with inorganic N addition. In addition, soil pH may have a role in determining N_2O yields by AOA. Currently, the highest measured N_2O yield for a cultured AOA is for the acidophilic I.1a-associated archaeon strain CS isolated from acid mine sediment (original pH 3.4, grown at pH 5.0), demonstrating ~5-50 times higher N_2O production than other agricultural strains isolated from soils with neutral pH (6.5–7.5) (Jung et al., 2014b). These results suggest a different N_2O production mechanism in acidic soils where AOA are probably the major contributors under oxic conditions. Acidic AOA might produce N_2O via the formation of nitrosonium cation (NO$^+$) from nitrous acid (HONO) under acidic conditions (Hughes, 1999). However, this process might rely more on abiotic hybrid via nucleophilic nitrosation of NH_2OH (Spott et al., 2011), and it still remains unclear what mechanism is used by acidophilic AOA. However, given the non-acidic pH of the soils in this study, the low AOA-associated N_2O yield is possible due to the possible absence of this abiotic hybrid process. The high yield of acidophile AOA strains is also possible due to their high sensitivity to nitrite, which can inhibit the growth of *Nitrosotalea devanaterra* and strain CS at 40 μM and 100 μM, respectively (Jung et al., 2014b; Lehtovirta-Morley et al., 2011), whereas *Nitrososphaera viennensis*, isolated from neutral pH soil, can tolerate nitrite at a concentration ~100 times more than that of *N. devanaterra* (Tourna et al.,

2011). Nevertheless, the detected N_2O in the headspace represented net N_2O accumulation, with N_2O consumption processes potentially occurring simultaneously. Denitrification is considered the major N_2O consumption pathway in soil (Hu et al., 2015; Vieten et al., 2008) and, although oxic conditions were maintained during microcosm incubation, it is possible that some microsites had low oxygen concentrations during the incubation period which facilitated denitrification activity. Subsurface soil samples may naturally possess higher denitrification activity due to low *in situ* oxygen concentrations and thus a greater denitrifier activity. In our previous research, we identified potential denitrifiers including *Pseudomonas* and *Thiobacillus* (Seuradge et al., 2017). Therefore, the absence of detected N_2O in some soils may be the result of relatively high denitrification activity resulting in the lowering of N_2O concentrations to less than the detection limit.

Seasonal (temporal) changes in N_2O production and nitrification were also detected in our soil samples. The summer samples showed a 10-fold higher N_2O yield than autumn soil samples, indicating the potential change of active populations in these soil samples. This difference might be due to different soil temperatures when we sampled the soils. The summer soil was 26 °C when sampled and the autumn soil was 14 °C; distinct ammonia oxidizer populations may have been selected during this time. Taylor and colleagues recently modeled temperature responses of AOA and AOB and demonstrated that AOA and AOB showed highest nitrification activity under different temperatures (Taylor et al., 2017). The optimal temperature for AOA was close to 40 °C for the soils samples they used, and AOB always showed highest activity below 30 °C, and sometimes below 20 °C. This range matched the soil temperature when we sampled, and may have explained the AOB-dominant activity difference between the two sampling points. However, identification of the active lineages in these soils under different temperatures would enable comparison their *in situ* nitrification activities to isolates or enrichments.

4.4. Influence of NO_2^- accumulation on soil N_2O production

Our results demonstrated a positive correlation between NO_2^- accumulation and N_2O production in surface agricultural soils during a 6-day incubation period (Fig. 5). Although this correlation was not significant for autumn soils, a strong positive correlation was found in soil samples collected from the same site in summer, indicating a possible seasonal effect on NO_2^- accumulation and nitrifier-associated N_2O production in these agricultural soils. An association between aerobic N_2O production and NO_2^- accumulation in soil has been observed in several studies (Duan et al., 2018; Giguere et al., 2017; Maharjan and Venterea, 2013; Venterea, 2007; Venterea et al., 2015). However, AOA- or AOB-dependent N_2O production was also observed in soils where NO_2^- was not detected (Hink et al., 2017). We observed both NO_2^--dependent and independent N_2O production in the same agricultural soils collected in different seasons, indicating a seasonal effect on AOA- or AOB-associated N_2O production. Indeed, both NO_2^--dependent and independent N_2O-producing mechanisms in ammonia oxidizers have been proposed in a number of studies (Cantera and Stein, 2007; Jung et al., 2014b; Kozlowski et al., 2014; Stieglmeier et al., 2014b), and nitrite-dependent N_2O production might be a detoxifying mechanism to protect nitrifiers from nitrite toxicity (Jung et al., 2014b). Moreover, growth of the recently discovered comammox bacterium *Nitrospira inopinata* can result in transient NO_2^- accumulation during complete oxidation of NH_3 (Kits et al., 2017), although the accumulated nitrite was subsequently converted to nitrate. Although it is likely that transient NO_2^- accumulation was the result of a temporary differences in the capacity between active ammonia and nitrite oxidizing populations, there is a possibility that our short-term incubation covered only the period of nitrite accumulation by comammox, if they were present in our soil samples. Given the possibility of an inhibition effect of octyne on comammox bacteria, we cannot exclude the possibility that nitrite accumulation which was detected only in soil samples with no added

octyne was produced by comammox bacteria in addition to AOB. Regardless of the NO_2^- source, our results confirmed that presence of NO_2^--dependent and independent N_2O production in the agricultural soils, and more importantly, the temperature-dependent nature of this mechanism.

4.5. Relative contributions of AOA and AOB to N_2O production

In our soils, AOB-associated N_2O production showed N_2O yields of $\sim 0.50\%$ for autumn samples and 2.43% for summer samples, a little higher than the range reported for *Nitrosomonas europaea* (Jung et al., 2011, 2014b), but within in the reported range of 0.02–7.6% (Hink et al., 2017; 2018; Jung et al., 2014b; Mørkved et al., 2007; Santoro et al., 2011; Shaw et al., 2006; Stieglmeier et al., 2014b; Zhu et al., 2013). The per cell AOB N_2O production rate (1.44×10^{-7} nmol cell^{-1} d^{-1} autumn, 8.84×10^{-7} nmol cell^{-1} d^{-1} summer) was calculated for the six-day incubation period, using the original AOB *amoA* gene abundance as an estimation. Considering that growth may have occurred during the six-day incubation, the actual N_2O-producing rate may be lower than the calculated value. Isolated representatives of *Nitrosospira*, which are typically the dominant soil AOB group, can have N_2O production rates of 0.9-1.4 $\times 10^{-7}$ nmol cell^{-1} d^{-1} (Smith et al., 2001), and the rate of *N. europaea* can be as high as 13.9×10^{-7} nmol cell^{-1} d^{-1} (Shaw et al., 2006). In general, AOB cultures have higher N_2O yields than AOA cultures, particularly when compared to marine AOA (Jung et al., 2011; 2014b; Santoro et al., 2011; Shaw et al., 2006; Stieglmeier et al., 2014b). This higher rate of N_2O production by AOB compared to AOA might be explained by AOB producing N_2O via two enzymatic mechanisms (i.e. the incomplete oxidation of hydroxylamine and nitrifier-denitrifcation) whereas it has been proposed that N_2O derived from AOA is only produced via the abiotic interaction of hydroxylamine and NO (Kozlowski et al., 2016). However, although Stieglmeier et al. (2014b) suggested that AOA may not be capable of nitrifier-denitrification, NO_2^--dependent N_2O production was measured during AOA-supported nitrification recently by Giguere et al. (2017), which raises the need for further investigation into NO_2^- stimulation of N_2O production.

5. Summary

Our results highlight differential temperature responses of AOA and AOB, with AOA dominating nitrification at higher soil temperatures. We identified AOB as major contributors to N_2O production through ammonia oxidation in the tested agricultural soil, influenced by temperature. Surface soils were major N_2O sources, whereas subsurface soils may not contribute significantly to soil N_2O production.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.

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